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jv809 U.S. PTO 04/18/00

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	MLY-5
First Inventor or Application Identifier	CHANG et al.
Title	MODIFIED FLUORESCENT PROTEINS FOR...
Express Mail Label No.	EL471679321US

jc564 U.S. PTO 09/551380 04/18/00

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231

1. [X] * Fee Transmittal Form (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. [X] Specification [Total Pages 33] *(preferred arrangement set forth below)*
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) *(35 U.S.C. 113)* [Total Sheets 7]
4. Oath or Declaration [Total Pages 40]
   - a. [X] Newly executed (original or copy)
   - b. [ ] Copy from a prior application (37 C.F.R. § 1.63(d)) *(for continuation/divisional with Box 16 completed)*
     - i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

5. [ ] Microfiche Computer Program *(Appendix)*
6. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
   - a. [X] Computer Readable Copy
   - b. [X] Paper Copy (identical to computer copy)
   - c. [X] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. [X] Assignment Papers (cover sheet & document(s))
8. [ ] 37 C.F.R.§3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney
9. [ ] English Translation Document *(if applicable)*
10. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
11. [X] Preliminary Amendment
12. [X] Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*
13. [X] * Small Entity Statement(s) (PTO/SB/09-12) [ ] Statement filed in prior application, Status still proper and desired
14. [ ] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
15. [X] Other: Express Mail certificate

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Prior application information: Examiner ______ Group / Art Unit: ______

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Address	P. O. Box 1449		
City	Greenville	State	SC
Zip Code	29602-1449		
Country	US	Telephone	864-271-1592
Fax	864-233-7342		

Name (Print/Type)	J. BENNETT MULLINAX	Registration No. (Attorney/Agent)	36,221
Signature	[signature]	Date	April 17, 2000

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Attorney Docket No.

Applicant or Patentee: Donald Choy Chang and Qian Luo
Serial or Patent No.:
Filed or Issued:
Title: Modified Green Fluorescent Proteins

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(b)) INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9 (c) for purposes of paying reduced fees under section 41 (a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled described in:

[X] the specification filed herewith
[ ] International application no.
[ ] filed

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Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

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[X] persons, concerns or organizations listed below*

FULL NAME: The Hong Kong University of Science & Technology
ADDRESS: Clear Water Bay, Kowloon, Hong Kong

[ ] Individual [ ] Small Business Concern [X] Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Donald Choy Chang

24 December, 1999

Date

Qian Luo

23-12-1999

Date

Attorney Docket No.

Applicant or Patentee: Donald Choy Chang and Qian Luo
Serial or Patent No.:
Filed or Issued:
Title: Modified Green Fluorescent Proteins

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF NONPROFIT ORGANIZATION:
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ADDRESS OF NONPROFIT ORGANIZATION:
Clear Water Bay, Kowloon, Hong Kong

TYPE OF NONPROFIT ORGANIZATION:

☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) AND 501(c)(3))

☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE:_____)
(CITATION OF STATUTE:_____)

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I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:

☒ the specification filed herewith with title as listed above.
☐ the application identified above.
☐ the patent identified above.

I hereby declare the rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights in the invention must file separate verified statements averring to their status as small entities and that no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization having any rights in the invention is listed below:

☒ no such person, concern or organization exists.
☐ each such person, concern or organization is listed below.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF PERSON SIGNING: Dr. Keith White-Hunt

TITLE IN ORGANIZATION OF PERSON SIGNING: Director of Business Development

ADDRESS OF PERSON SIGNING: Hong Kong University of Science & Technology, Clear Water Bay, Kowloon

SIGNATURE: _____ DATE: 13 DEC 1999

PATENT

ATTORNEY DOCKET NO.: MLY-5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of DONALD CHOY CHANG et al.	)	Examiner: (Unknown)
Serial No.: (Not assigned)	)	Art Unit: (Unknown)
Filed: (Herewith)	)	
For: MODIFIED FLUORESCENT PROTEINS FOR DETECTING PROTEASE ACTIVITY	)	

Assistant Commissioner
for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Dear Sir:

Please enter the following first preliminary amendment in the above-referenced application filed herewith.

IN THE CLAIMS:

Please amend claim 27 as follows.

27. (Amended) A method according to [any one of claims 11-15,] claim 11, said protease comprising a caspase and said method comprising a method of detecting apoptosis of said sample cell.

REMARKS

By way of the above first preliminary amendment, applicant has amended claim 27 in the above application to remove the multiple dependency and, more specifically, to conform with the filing requirements in the United States.

Respectfully submitted,

DORITY & MANNING, P.A.

J. BENNETT MULLINAX
Registration No. 36,221

P. O. Box 1449
Greenville, SC 29602-1449
Telephone: (864) 271-1592
Facsimile: (864) 233-7342

Modified Fluorescent Proteins for Detecting Protease Activity

FIELD OF THE INVENTION

The present invention concerns modified fluorescent proteins, particularly modified green fluorescent proteins (GFPs), having a protease cleavage site whose cleavage causes the alteration of one or both of the emission and excitation spectra of the fluorescent protein. It also concerns methods for detecting protease activity and agents which affect same.

BACKGROUND OF THE INVENTION

Fluorescent proteins, particularly green fluorescent proteins, and their uses are well known in the art. US 5491084 discloses various uses of a green fluorescent protein, together with host cells having gene constructs encoding a GFP, and methods for selecting cells expressing a protein-of interest. US 5625048 and US 5777079 disclose modified GFPs having emission and excitation spectra different to those of wild-type GFPs. US 5804387 discloses GFP mutants having modified excitation and emission spectra.

Determining *in vivo* protease activity is extremely desirable since proteases can have a significant effect upon cellular events. For example, the early stage of apoptosis (programmed cell death) is signified by protease (caspase) activity and so an assay for appropriate protease activity can be an assay for apoptosis. Apoptosis is an induced cell suicidal process that allows the biological organism to destroy damaged or unwanted cells in an orderly way (Kerr, J.F.R. *et al.*, 1972, Br. J. Cancer, 26: 239-257), and because of this it is a very important cellular process. It plays a vital role in maintaining the normal physiological function in a variety of ways. For example, the process of apoptosis is used in the thymus to eliminate self-reactive T cells to avoid auto-immunity (Thompson, C.B., 1995, Science, 267: 1456). Furthermore, when DNA is damaged in

a cell and cannot be repaired, the cell will enter apoptosis to avoid the formation of abnormalities in the tissue. Thus, failure of programmed cell death can cause cancer. On the other hand, excessive apoptosis can also cause great damage to the body; it is linked to many neural degenerative diseases such as Huntington disease and Alzheimer's disease.

In the last few years, a large number of studies have been conducted aiming to understand the process of apoptosis on a molecular basis. The signalling pathways that direct the programmed cell death process turns out to be very complicated (Ashkenazi, A. and Dixit, V.M., 1998, Science, 281: 1305-1308; Thornberry, N.A. and Lazebnik, Y., 1998, Science, 281: 1312-1316; Evan, G. and Littlewood, T., 1998, Science, 281: 1322-1326; Adams, J.M. and Cory, S., 1998, Science, 281: 1317-1322). There are many external signals that can trigger the initiation of apoptosis, including UV-irradiation, activation of the "death domain" *via* the TNF (tumour necrosis factor) receptor or CD95, treatment with hormones (e.g. glucocorticoid) or chemotherapy drugs (e.g. camptothecin) (Ashkenazi, A. and Dixit, V.M., 1998, *supra*; Nagata, S., 1997, Cell, 88: 355-365; Martin, S.J. and Cotter, T.G., 1991, Int. Radiat. Biol., 59: 1001-1016). As for the internal signals, it is known that apoptosis is the outcome of a programmed cascade of intracellular events, which are centred on the activation of a class of cysteine proteases called "caspases" (Thornberry, N.A. and Lazebnik, Y., 1998, *supra*). At present, the detailed molecular mechanisms by which apoptosis is regulated by the various internal and external signals are still not well understood.

The process of apoptosis can be detected at different stages. For example, some apoptosis assays are based on events that occur rather late in apoptosis, such as morphological changes of the cell, nuclear breakdown, and chromosomal fragmentation. Some assays can detect relatively early events such as the turn-over of certain phospholipids in the membrane (Martin, S.J. *et al.*, 1995, J. Exp. Med., 182: 1545-1555).

Alternatively, one can assay the activation of caspase-3 based on the fact that substrates of caspase-3 have a specific sensing and cleavage sequence (Nicholson, D.W., 1996, Nature Biotechnol., 14: 297-301). By linking a peptide encoding this substrate sequence to a fluorescent dye, one can detect a shift of the fluorescent properties of the dye when the peptide is cleaved by the activated caspase (CLONTECHniques, 1997, 12(1): 4-6). The activity of caspase-8 can be similarly assayed (www.clontech.com, ApoAlert Caspase Assay Kits). Since these methods utilize an optical detection, they are simple and quick. These methods do, however, have certain limitations. For example, the probes cannot penetrate the cell membrane, thus it is difficult to load the probes inside the cell. The assays are therefore done using crude cell lysates, i.e. not with whole (living) cells and not *in vivo*. Furthermore, the fluorescent change resulting from caspase cleavage involves mainly a shift of the emission spectrum (from blue to yellow-green) rather than an easier to assay total destruction of the fluorescence. In addition, its sensitivity is limited.

BRIEF SUMMARY OF THE INVENTION

The present inventors have succeeded in providing a simple, accurate, easy-to-assay system for determining protease activity, particularly caspase activity, which works both *in vivo* and *in vitro*.

According to the present invention there is provided a fluorescent protein modified such that said modified fluorescent protein incorporates a cleavage site for a protease, cleavage of said modified fluorescent protein at said cleavage site by said protease causing the alteration of at least one of the emission and excitation spectra of said modified fluorescent protein. In particular, the invention is concerned with modified green fluorescent proteins.

Also provided are nucleic acid sequence encoding same, recombinant DNA constructs expressing same, cells transformed or transfected with same, methods for detecting protease activity, and methods of detecting agents which affect protease activity, and kits for same.

Although particularly useful in detecting caspase activity, the invention also extends to modified fluorescent proteins cleavable by other proteases to alter at least one of its emission and excitation spectra.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows bacterial colony expression of the wild-type GFP (top) and the GFP-DEVD mutant (bottom) on LB agar plate;

Figure 2 shows Western blot analysis of caspase-3 cleavage using an anti-GFP antibody. Cell extracts of wild-type GFP clone (lanes 1 and 2) and GFP-DEVD mutant (clone D9) (lanes 3 and 4) were treated with (+) or without (-) caspase-3. Only the D9 mutant was cleaved by the caspase-3 treatment. The protein band with a smaller molecular weight seen in lane 4 represents the major cleavage fragment of GFP;

Figure 3 shows phase contrast and fluorescent images showing the change of fluorescent intensity in HeLa cells transfected with wild-type GFP and mutant GFP (MD9) before and after the induction of apoptosis by TNFα treatment;

Figure 4 shows relative fluorescence intensity of mutant GFP (MD9) in comparison to the wild-type GFP, measured as a function of time after HeLa cells were induced to enter apoptosis by a TNF treatment. Data is the average of four cells;

Figure 5 shows the principle of design of the intra-GFP probes of the present invention. A short peptide containing the substrate sequence of the caspase (referred to as "sensor") is inserted into the intra-molecular region of the GFP molecule. When caspase is activated, it cleaves the sensor and destroys the fluorescence properties of the GFP;

Figure 6 shows Western blot analysis of MD9 mutant during TNF induced apoptosis. HeLa cells expressing either enhanced GFP (EGFP) or a mammalian version of D9 mutant (MD9) were treated with 5 ng/ml TNFa and 5 μg/ml of cycloheximide for 0, 1, 2, 4 and 6 hours. Cell extracts were analysed by Western blot. It is evident that only the MD9 mutant protein (but not EGFP) was cleaved during apoptosis; and

Figure 7 shows the locations of mutation sites in GFP as shown in its 3-D structure. The mutation sites of D4, D7, D8 and D9 are all in the loop regions between different beta sheets. More specifically, mutant sites D4 and D8 are between the beta sheets No.5 and No.6. The site for mutant D7 is between beta sheets No.8 and No.9, and the site for D9 mutant is between beta sheets No.9 and No.10. The 3-D structure of GFP is based on the paper of Yang F., *et al.*, 1996, Nature Biotechnology, 14:1246-1251.

DETAILED DESCRIPTION OF THE INVENTION

The gene for GFP was originally isolated from the jellyfish *Aequoea victoria* (Prasher, D.C. *et al.*, 1992, Gene, 111: 229-233). Its gene product contains a spontaneously formed chromophore generated through an oxygen-dependent cyclization reaction involving three amino acids (residue 65-67) (Cubitt, A.B. *et al.*, 1995, TIBS, 20: 448-455). This gene can be expressed in cells of many different biological systems to produce an endogenous fluorescent protein without the requirement of adding exogenous substrates or coenzymes (Cubitt, A.B. *et al.*, 1995, *supra*; Chalfie, M. *et al.*, 1994,

Science, 263: 802-805). In the last several years, GFP has been used widely in cell and molecular biology. For example, GFP has been used as reporters of gene expression, tracers of cell lineage, and fusion tags for monitoring protein localization within living cells (Cubitt, A.B. *et al.*, 1995, *supra*; Gerdes, H.H. and Kaether, C., 1994, FEBS Lett., 384: 44-47).

The experiments below detail the production of modified green fluorescent proteins having caspase-specific recognition sites, the modified green fluorescent proteins being cleaved by the caspase enzymes only at the caspase-specific recognition sites. The efficacy of these recognition sites in enabling cleavage which affects fluorescence in the presence of a caspase specific against them varies between the differently modified GFPs, and the best modified protein produced so far is D9 (below) which is cleavable by a caspase and displays the greatest change in fluorescence (i.e. change in emission and/or excitation spectra) upon cleavage. Other useful modified proteins which can be cleaved by a caspase resulting in a change in fluorescence are D4, D7 and D8. The experiments show cleavage by caspase-3. Recognition sequences for other proteases are well known (see for example Table 3), and so can be readily incorporated into a modified fluorescent protein, for example in a modified GFP at the sites used to make D9, D4, D7 or D8 (see below). The sequences of SEQ ID NOs: 8-10, 12 and 13 contain optimal sequences for caspase recognition and cleavage and are artificial sequences defined by a positional screening of a tetrapeptide library for their caspase substrate specificity (Thornberry, M.A. *et al.*, 1997, J. of Bio. Chem., 272: 17907-17911). More generally, modifications to incorporate a cleavage site can be at the loop structures of fluorescent proteins such as GFPs joining β-sheets, for example GFP β-sheets numbers 9 and 10, 5 and 6, or 8 and 9, the β-sheets being exposed on the exterior of the fluorescent protein.

As well as providing modified fluorescent proteins, nucleic acid sequences encoding same are also provided and are readily derived from the amino acid sequences of the

proteins. The nucleic acid sequences encoding the proteins are used to produce recombinant DNA constructs additionally comprising a regulatory element operatively linked to the nucleic acid sequence, the regulatory element directing (i.e. being sufficient to drive) expression of the protein coded for. Regulatory elements are widely known and include those which cause constitutive expression, and those which are triggered by specif events or stimuli. Also provided are cells transformed or transfected with such recombinant DNA constructs. Such cells are particularly useful since they provide for the *in vivo* assay of protease levels. Techniques useful in achieving this are well known in the art and are described by e.g. Sambrook, J., Frisch, E.F., and Maniatis, T., "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York, 1989; "PCR (Volume 1): A practical approach" Eds. M.J. McPherson, P. Quirke and G.R. Taylor. Oxford University Press, 1991; "General Techniques of Cell Culture", Harris, M.A. and Rae, I.F., 1997, Cambridge University Press, ISBN 0521 573645; Huynh and Davies, 1985, "DNA Cloning Vol I - A Practical Approach", IRL Press, Oxford, Ed. D.M. Glover.

The proteins of the invention are useful in a number of applications, particularly assays for determining levels of protease activity in a sample, in determining changes in protease activity in a sample and in determining whether specific compounds or other substances affect protease activity in a sample. Specific samples include living cells - sample cells having a protease activity to be determined and control cells having a known protease activity which are used in assays to determine protease activity in the sample cell with reference to the known protease activity in the control cell. In detecting changes in protease activity, only the sample cell is needed with observations of fluorescence at different timepoints allowing changes in protease activity to be determined. Using at least three timepoints allows the rate of change to be determined, and can be standardised by reference to changes in a control sample having a known

protease activity. Control samples can simply have no protease activity. Samples can also of course be other than living cells, for example crude cell lysates.

Substrates of a specific caspase often share a consensus recognition and cleavage amino acid sequence (Nicholson, D.W., 1996, *supra*). For example, the recognition sequence for caspase-3 is SEQ ID NO: 4. Using genetic engineering techniques, a molecular probe to assay the caspase-3 activity was produced by inserting the gene encoding its recognition/cleavage sequence into the gene encoding GFP. When this mutated GFP is expressed in a cell, its gene product (i.e. a modified GFP protein containing the specific caspase-3 recognition/cleavage sequence) is cleaved by the caspase upon its activation. The caspase-3 site can readily be replaced by the recognition and cleavage site of another caspase such as caspase-8 or caspase-9 (see Table 3). It can also be replaced by the cleavage site of any other protease and so can allow for the assaying of the activity of any chosen protease. The term "cleavage site" is used herein to refer to both the recognition and cleavage sites of a protease.

As discussed above, caspase activity in a cell, particularly caspase-3 activity, correlates with the start of programmed cell death (apoptosis). This means that a particularly useful assay is that of caspase-3 activity in order to determine the onset of apoptosis, its progress, and also to identify whether given compounds, substances or compositions (herein "compounds") have an effect upon apoptosis. For example, this can allow the detection of agents which trigger the start of apoptosis or hinder it, or in the case of patients such as certain cancer patients having cancer cells which are unable to undergo apoptosis, it allows the detection of agents which can re-enable apoptosis in such cells. Similarly this can also allow the identification and testing of first compounds which modify the apoptosis-inducing ability of second compounds, for example the second compound could be an environmental poison and the first compound an inhibitor of that poison.

In particular, *in vivo* assays can be performed using a continual analysis of excitation/emission spectra of a sample to determine the exact point at which apoptosis begins, as determined by caspase-3 activity. This resolution in the time domain has not previously been possible using e.g. cell lysates since they require time-consuming steps in order to perform an assay starting with complete sample cells/ In addition such assays are much less resource-intensive since a single preparation of a cell or cells can be used at many time-points instead of just one. Regarding the analysis of the excitation and/or emission spectra, this can be done with entire spectra or by "windowing" i.e. by analysing only a specific frequency range or ranges within the emission and/or excitation spectra. For example, this can be done using filters for incident and emitted light, which isolate a specific range of wavelengths. Similarly, when cleavage results in an overall destruction of fluorescence, the fluorescence intensity of a sample may be assayed, without reference to specific frequencies. Reference herein to determining, comparing and correlating emission and/or excitation spectra therefore includes determining, comparing and correlating the emission and/or excitation spectra at specific frequencies or ranges of frequencies, as well as overall fluorescence intenesity.

The invention also extends to fluorescent proteins other than GFP, and in particular concerns BFP (blue fluorescent protein), CFP (cyan fluorescent protein), YFP (yellow fluorescent protein) - fluorescent proteins available from e.g. Clontech and PharMingen - and DsRed (a red fluorescent protein isolated from *Discosoma striata* - Matz, M.V. *et al.*, Nature Biotechnol., 17: 969-973) modified such that they incorporate a cleavage site for a protease, such that cleavage of said modified fluorescent protein at said cleavage site by said protease causes the alteration of at least one of the emission and excitation spectra of said modified fluorescent protein. The loop structures of the fluorescent protein which join β-sheets and which are exposed on the exterior of the fluorescent protein are particularly good sites for modification to incorporate a cleavage site.

Examples

In the design of our molecular probe, we place the caspase recognition sequence into specific intra-molecular locations of the GFP such that (i) the insertion does not destroy the fluorescent properties of the GFP, and (ii) the substrate is well accessible to the protease. Since GFP is known to have a very "tight" structure, the possible insertion locations that can satisfy both of these conditions are limited. X-ray diffraction studies have indicated that the crystal structure of GFP is an 11-stranded β-barrel with a coaxial α-helix, with the chromophore forming from the central helix (Ormo, M. *et al.*, 1996, Science, 273: 1392-1395; Yang, F. *et al.*, 1996, Nature Biotechnol., 14: 1246-1251). The major candidates for insertion are the loops between the various adjacent β-sheets, in particular the loops between the β-sheets numbers 5 and 6 (D4 and D8), 8 and 9 (D7), and 9 and 10 (D9). Also, in order to avoid a significant change of protein folding, the mutation must have minimal disturbance on the charge properties of the protein at the insertion site.

With this new molecular probe, activation of apoptosis can be assayed easily in living cells. The typical procedure of using this method to detect apoptosis is:

1. The plasmid DNA containing a modified GFP gene is introduced into host cells by standard gene transfer methods, such as electroporation, calcium phosphate or lipofectin.

2. When the transferred gene is expressed, the presence of the gene product (i.e. the modified GFP protein) can be detected by its green fluorescence using an optical device, such as a fluorescence microscope, a fluorescent activated cell sorter (FACS), or a fluorometer.

3. The transfected cells are then ready for induction to enter apoptosis. Depending on the purpose of the experiment, apoptosis can be induced by a number of means, such as treating the cells by TNF, UV or glucocorticoid.

4. When the caspase-3 is activated during apoptosis, it cleaves the modified GFP molecule at the substrate insertion site. This cleavage results in a dramatic change of the fluorescent properties of the GFP, which can be easily detected using an optical device (above) such as a fluorescence microscope. In this way, one can easily assay whether or not a particular cell or a population of cells are undergoing programmed cell death.

Furthermore, using a stable transfection technique, the gene encoding the molecular probe can be permanently inserted into the chromosomes of the target cells. In such a way, stable cell lines can be generated that can automatically generate the mutated GFP probe. Such cell lines can then be used of *in vivo* assay of apoptosis. These cell lines are particularly useful for rapid screening of drugs or for toxicity testing.

Example 1

MATERIALS AND METHODS

Cell lines and culture media

Escherichia coli BL21-DE-3 (Invitrogen, CA, USA) and DH5α (Life Technologies Inc., USA) were grown either in liquid LB (Luria-Bertani) medium or on a solid LB agar plate. The Oxoid tryptone and yeast extract for preparing LB culture media were obtained from Unipath Ltd. (Basingstoke, Hampshire, England). The select agar for making LB agar plates was obtained from Life Technologies Inc. (Paisley, Scotland).

The HeLa cells were grown in MEM (Minimum Essential Medium) supplemented with 10% FBS (Fetal Bovine Serum), 100 U/ml penicillin, 100 U/ml streptomycin (Life Technologies Inc., Grand Island, New York, USA) and 0.37% $NaHCO_3$ (BDH Laboratory Supplies, Poole, England).

Modified Site-Directed Mutagenesis

The essential procedures of site-directed mutagenesis are described in the following: Two complementary oligonucleotide primers (see for example SEQ ID NOs: 1 and 3) containing DNA sequence encoding amino acids of SEQ ID NO: 4 in the middle of the primer and 13-17 bp of GFP gene specific sequence on both sides of the SEQ ID NO: 4 sequence were synthesized and cleaned up using QIAquick Nucleotide Removal Kit obtained from QIAGEN (Germany). A double-stranded plasmid containing the gene encoding GFP(S65T) (SEQ ID NO: 5; Heim, R. *et al.*, 1995, Nature, 373: 664-664) was used as a template in the mutagenesis reaction, which comprised the following steps:

(1) The template was first denatured at 96 °C for 1-2 minutes

(2) The mutagenic primers were allowed to bind to the single-stranded DNA template at an annealing temperature

(3) Using a high fidelity Pwo DNA polymerase obtained from Boehringer Mannheim (Mannheim, Germany), the primers were extended at 68 °C for 10 minutes to synthesize a nicked circular plasmid

A total of 18-20 cycles of the reaction were performed. After the amplification reaction, the nonmutated parental DNA of GFP(S65T) was digested by *DpnI* endonuclease that specifically digests methylated and hemimethylated DNA produced by bacteria. The nonmethylated nicked plasmids, newly generated by primer extension, were transferred into the bacteria host *E.coli* BL21-DE-3 cells, which ligate the mutated plasmids into circular plasmids and amplify them. These mutated plasmid DNA were isolated from the

bacteria using High Pure Plasmid Isolation Kit obtained from Boehringer Mannheim and subjected to further analysis.

23 GFP mutants were generated using this modified site-directed mutagenesis method and are detailed in Tables 1 and 2. 19 of the GFP mutants were constructed using the GFP(S65T) gene (below; Table 1), whilst 4 were generated by mutating the EGFP gene (Table 2) which is optimised for expression in a mammalian host. The experimental condition for each primer extension reaction was adjusted according to the type of the mutation, such as single amino acids insertion or multiple amino acids insertion or replacement.

The following example shows a detailed procedure for constructing one of the GFP mutants, D9. Other mutants (i.e. modified GFPs) were similarly generated but resulted in different modifications.

Generation of D9 mutant

In this experiment, three amino acids (EVD) were inserted into GFP(S65T) at the position between D_{190}--G_{191} (see Table 1) in the following steps:

1). Two complementary mutagenic primers (KL35 and KL36) were synthesized by Life Technologies (Pacific) Ltd., and further purified using QIAquick Nucleotide Purification Kit (from QIAGEN) according to the manufacturer's protocol. The DNA and amino acids sequences for the 5' primer (KL35) are SEQ ID NOs: 1 and 2 respectively. KL36 is SEQ ID NO: 3.

2). Prepare the sample and control reactions on ice as indicated below:

Components	Sample	Negative Control
10x reaction buffer	5 µl	5 µl
10 mM dNTPs	1 µl	1 µl
5' primer (KL35) (10 pmol/µl)	3 µl	3 µl
3' primer (KL36) (10 pmol/µl)	3 µl	3 µl
dsDNA template (10 ng/µl)	5 µl	0
Double-distilled water (ddH_2O)	33 µl	38 µl

Mix all the components and add 0.5 µl of Pwo DNA polymerase (5 U/µl) (from Boehringer Mannheim) into each tube. Put the reaction tubes into the PCR machine when the temperature of the PCR machine is higher than 85°C.

3). Run the reaction in the following temperature cycling:

Steps	Function	Temperature and Time
1	pre-denaturation	at 96 °C for 2 minutes
2	denaturation	at 96 °C for 1 minute
3	annealing	at 55 °C for 1 minute
4	extension	at 68 °C for 9 minutes
5	repeat steps 2-4 for 17 more cycles	
6	final extension	at 72 °C for 15 minutes
7	cooling	at 4 °C for 5 minutes
8	end	

4). Purify the PCR product from primers, nucleotides, polymerase and salts using QIAquick PCR Purification Kit (from QIAGEN). Elute the DNA with 45 µl of ddH_2O in a microcentrifuge tube.

5). Add 2 µl of the *DpnI* restriction enzyme (6 U/µl, from Life Technology) and 5 µl of 10x *DpnI* reaction buffer to each purified PCR product. Mix each reaction thoroughly and incubate them at 37°C for 2 hours to digest the methylated and nonmutated parental DNA template.

6). Heat at 65°C for 15 minutes to inactivate the *DpnI* restriction enzyme.

7). The circular, nicked and double-stranded DNA (dsDNA) is separated from other non-specifically amplified DNA by electrophoresis through a 0.8% agarose gel (from Boehringer Mannheim).

8). The DNA with the correct size is excised from the agarose gel and extracted from the gel slide using QIAquick Gel Extraction Kit (from QIAGEN). Elute the DNA with 40 µl of ddH_2O.

9). Transform 100 µl of competent *E.coli* BL21-DE-3 cells with 4 µl of DNA and select the colonies harboring the mutated pRSET-GFP plasmid (Heim, R. *et al.*, 1995, Nature, 373: 663-664) on LB agar culture plates containing 100 µg/ml ampicillin (Sigma, St. Louis, MO, USA).

Prepare bacterial cell extracts containing the mutated GFP

1). A single green fluorescent colony was inoculated into 3-5 ml of LB medium containing ampicillin (100-200 µg/ml) in the morning. Cells were grown at 37°C with shaking at 200 rpm/min until OD_{600} = 0.7-0.9.

2). 0.4 mM IPTG (Promega, Madison, WI, USA) was added into the cell culture to induce GFP expression at room temperature overnight. In addition, 200 µg/ml

ampicillin was added to the cell culture to prevent plasmid loss during this long period of induction.

3). Cells were collected by centrifugation at 4,800 rpm for 5-10 minutes and washed once with 15 ml of cold PBS. Cell pellets were resuspended in 0.3 ml of caspase assay buffer D containing 20 mM HEPES (from Life Technologies), 10 mM KCl (from Sigma), 5 mM DTT (from Boehringer Mannheim), 1 mM EDTA (from Sigma) and 0.1% CHAPS (Merck, Darmstadt, Germany), pH 7.2.

4). Cells were partially lysed by freezing in a dry ice/ethanol bath and thawing in a cold water bath. This process was repeated three times.

5). Cells were further lysed by sonication on ice for at least three times at 1 minute bursts / 1 minute cooling at the maximum strength of the sonicator (Labsonic U).

6). Soluble proteins were separated from cell debris by centrifugation at 14,000 rpm for 10 minutes at 4 °C. The clear supernatant was transferred into a microcentrifuge tube and stores at -20 °C.

Characterization of GFP mutants by caspase-3 assay, fluorescence measurement and Western blot analysis

10 µl of cell lysate prepared from each GFP mutant was mixed completely with 2.5 µl of active caspase-3 (20 ng/µl) (PharMingen, San Diego, CA, USA) and 37.5 µl of the caspase assay buffer D. The reaction was carried out at 37 °C for 2 hours.

Each reaction mixture was then transferred into each well of a 96 well plate. The fluorescence intensity was measured using a fluorescence plate reader, CytoFluorII

(Bioresearch, USA) with an excitation wavelength range of 468-492 nm and an emission wavelength range of 510-540 nm.

To perform Western blot analysis, 20 µl of the reaction mixture was mixed with 6 µl of 4x SDS page sample buffer containing 200 mM Tris-HCl, pH 6.8, 40% glycerol, 0.4% bromophenol blue (all from Sigma), 8% SDS (Riedel-de Haën), and 10% β-Mercaptoethanol (Fisher Scientific, Fair Lawn, New Jersey, USA). Protein samples were boiled for 5 minutes and subjected to SDS-PAGE on 12% or 15% gels. Proteins were subsequently electrotransferred onto a Hybond ECL nitrocellulose membrane (Amersham Life Science Ltd., Little Chalfont, Buckinghamshire, England). Protein blot was blocked with 5% non-fat dry milk and probed with polyclonal anti-GFP antibody at dilution of 1:5,000 (Molecular Probes Inc., Eugene, Oregon, USA) for 1-2 hours at room temperature. The membrane was washed 3-4 times for 5 minutes in TBS with 0.2% Tween-20 (from Sigma). The immunoblot was then probed with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody at dilution of 1:5,000 (Bio-Red, Hercules, CA, USA). Finally, the proteins were detected using the ECL (RTM) western blotting analysis system (from Amersham).

Generation of MD9 mutant

A mammalian version of the EGFP mutant was generated usin the plasmid DNA of EGFP-C3 (Clontech, Palo Alto, CA) as a template in the mutagenesis experiments. The construct was prepared by following the procedures described above in the Materials and methods section under the heading "*Generation of D9 mutant*" from step 1 to step 9.

Generating stable mammalian cell lines expressing GFP-DEVD recombinant protein

Using a stable transfection technique (below), the gene encoding GFP-DEVD (Table 2, MD9) is permanently integrated into the chromosomes of the target cells. In such a way, stable cell lines are generated that can automatically produce this intra-GFP probe. Such

cell lines can then be used for the *in vivo* assay of apoptosis. These cell lines are particularly useful for rapid screening of drugs or for toxicity testing. The detailed procedure for generating such stable cell lines is described below:

(1) The plasmid DNA of EGFP mutants (see Table 2) containing an engineered caspase-3 cleavage site (SEQ ID NO: 4) waslinearized by restriction digestion with EcoRI (Boehringer Mannheim). The position of the cleavage is within the multiple-cloning site region of the vector.

(2) 2-4 μg linear plasmid DNA was introduced into $5x10^5$ attached mammalian cells (e.g., HeLa) by electroporation.

(3) Cells were seeded into a 60-mm culture dish, and incubated at 37°C with 5% CO_2 for 24-48 hours to allow the EGFP-DEVD mutant to be expressed.

(4) The efficiency of electroporation was determined by visualizing and counting the percentage of fluorescence positive GFP-DEVD cells under a fluorescence microscope.

(5) The cells were trypsinized and plated at dilution of 1:25 to 1:40 in the presence of 0.3-1.0 mg/ml antibiotic G418 (from Life Technologies). The concentration of G418 was determined by performing a kill curve with each particular cell line at a defined cell density.

(6) The selection medium was changed every 3 days for 14-20 days. Individual colonies exhibiting green fluorescence under a dissecting GFP microscope (*Leica* MZ12) were selected and expended into clonal cell lines.

(7) Genomic DNA from cells of the selected stable cell line was isolated and analyzed by Southern blot analysis to confirm the success of chromosomal integration of the GFP-DEVD gene.

RESULTS

a. We have generated mutated GFPs (above) with an insertion of a sensor sequence. These mutated GFP can retain the endogenous fluorescent properties.

The expression of the green fluorescent protein from each bacterial colony was examined under a fluorescent microscope (Leica MZ12) with excitation and emission filters for GFP. Green fluorescent signal was observed from various of the GFP mutants (Tables 1 and 2). The fluorescent intensity of some of the mutated GFP-DEVD was weaker than that of the wild-type GFP, but can still be detected clearly (Figure 1).

b. These mutated GFP can be cleaved by caspase-3 as evident from Western blot assays.

We then performed caspase assay on GFP mutants of D4, D7, D8, and D9 to determine which mutant can be cleaved by caspase-3. The cell extracts from each mutant were incubated with purified caspase-3 (PharMingen) at 37°C for 2 hours, and then analyzed by Western blot assay using an anti-GFP polyclonal antibody (Clontech). The results are shown in Figure 2. The GFP antibody interacted specifically with two bands with molecular weight corresponding to the poly-histidine tagged GFP and its degradation product from cells transfected with either GFP or mutant D9 (lanes 1 and 3). No proteins at the same molecular weights were detected by the GFP antibody from control cells expressing only the pRSET vector (data not shown). These results showed that the GFP antibody can detect both the wild type and the mutated GFP protein. When caspase-3 was added to the cell extract, the protein band of untagged GFP-DEVD disappeared

while a new protein band representing the cleaved product emerged (lane 4), indicating that the mutant GFP was indeed cleaved by caspase-3. On the other hand, no cleavage occurred for the wild type GFP in the presence of caspase-3 (lane 2). Results of this *in vitro* assay clearly showed that the substration site (DEVD) inserted in the D9 mutant could be recognized and cleaved by the caspase-3. Similar Western blot analysis of cell extracts containing GFP mutants D4, D7 and D8 showed that those mutant proteins could also be cleaved by the caspase-3 (Tables 1 and 2).

c. When the mutated GFP was expressed in cultured mammalian cells, its fluorescent intensity was shown to decrease when cells entered apoptosis.

To demonstrate that this newly developed mutant GFP probe can indeed be used in an *in vivo* assay of apoptosis, we have introduced plasmid DNA of both wild-type GFP (pEGFP-C3; SEQ ID NO: 32) and mutant GFP (MD9, the mammalian version of D9) into HeLa cells by electropration. The cells were seeded to a 35 mm-petri dish with a glass coverslip attached to the bottom, and cultured in a humidified CO_2 incubator at 37 °C. At 30 hours after electroporation, the petri dish was transferred to a micro-incubation chamber (37 °C) and mounted onto the stage of an epi-fluorescence microscope. TNFα (5 ng/ml), together with cycloheximide (5 μg/ml), was applied to the cells to induce apoptosis. Fluorescent images of the cells expressing the fluorescent protein were recorded both before and after the induction of apoptosis. The fluorescent intensity inside the cells was measured using a computer-controlled imaging system (Li, C.J. *et al.*, J. Cell Science, 112(10): 1567-1577). We found that the fluorescent intensity of cells transfected with mutant GFP (MD9) decreased significantly when cells entered apoptosis (Figure 3). The relative fluorescence intensity of mutant GFP in comparison to wild type GFP was found to reduce by about 50% six hours after the TNFα treatment (Figure 4). These results indicated that the fluorescent intensity of the mutant GFP, which has an

insertion of caspase substrate (SEQ ID NO: 4) was indeed responsive to the activation of caspase-3 during apoptosis.

d. Western blot analysis of MD9 cleavage in TNF-induced apoptosis of HeLa cells

The plasmid DNA of MD9 and EGFP-C3 were transfected into HeLa cells, respectively, by electroporation. The cells were then seeded to 60 mm petri dishes and cultured in CO_2 incubator. After 48 hours of transfection, TNF (5 ng/ml), together with cycloheximide (5 μg/ml), was applied to the medium to induce apoptosis in these transfected cells. The samples were collected at 0, 1, 2, 4 and 6 hours after TNF-application, respectively, to perform Western blot analysis. When harvesting the cells, a plastic policeman was used to scrape the monolayer from the plate of the dish. The scraped cells were then transferred to a 15 ml centrifuge tube, together with the culture medium. The samples were centrifuged at 1500 rpm for 2 minutes. The pellet was washed with PBS once. Then 50 μl 1% SDS (in 10 mM Tris-HCl, pH 7.4) was applied to the pellet to lyse the cells. The cell lysate was centrifuged at 14,000xg for 3 minutes at 4 °C. Then the supernatant was used to conduct Western blot analysis against the rabbit polyclonal antibody of anti-GFP (1:1000) from Molecular Probes, Inc. The result showed that the mutant GFP protein could be cleaved by activated caspase-3 in a time-dependent manner during the process of apoptosis. It can be seen from the figure that more than 50 % of the mutant protein had been cleaved into smaller fragments after 4 hours of TNF-treatment. No cleavage had been seen in wild-type GFP during the process of apoptosis.

Example 2

To specifically determine protease (e.g. caspases) activity in sample cells of Example 1, the sample cells have their emission and/or excitation spectra determined. This is then compared to the same emission/excitation spectra determined for cells of Example 1

having a known protease activity, and the results then correlated to determine protease activity in the sample cells.

Example 3

To determine a change in protease (e.g. caspase) activity, the fluorescent intensity of sample cells is determined at a first timepoint using an imaging device. At a second timepoint the same fluorescent image is determined for the same cells. The results at the first and second timepoints are then compared and correlated to determine any change in protease activity.

To determine the rate of change of protease activity, at least one more timepoint must be added. Results can be standardised by comparing them to similarly obtained results for cells having a known protease activity.

Example 4

To determine the effect of a compound or substance/composition on the activity of a protease in a cell or cells of Example 1, the method of Example 3 is employed but with the compound being administered to the cell between the first and second timepoints, the final correlation determining the effect of the compound upon the cell.

The method can be supplemented by comparison to similarly obtained results for a cell or cells having a known protease activity.

Example 5

To determine the effect of a compound or substance or composition on cells of Example 1, the method of Example 2 is employed but with the sample cells having first been treated with the compound, the final correlation determining the effect of the compound on the sample cells.

Example 6

Examples 4 and 5 concern the effect of one coumpound or substance/composition on the activity of a protease in a cell and on the cell. The effect of first and second compounds, the first compounds modify the apoptosis-inducing ability of the second compound, upon proteases and cells is determined by administering the first and second compounds at the same or different timepoints during the methods of Examples 4 and 5.

Example 7

The methods of Examples 2-6 need not be performed on cells and can instead be performed using mixtures of the necessary reagents (i.e. in a cell-free environment). As discussed above, GFP does not need any additional reagents such as ATP to fluoresce. Thus a mixture comprising a modified GFP of the present invention, chosen protease and the reagents and conditions necessary to allow fluorescence of the modified GFP are sufficient to allow an assay of protease activity. Other reagents and test compounds/compositions may also be included in the mixture to allow an analysis of their effect upon protease activity or fluorescence.

Example 7

An assay for determining the start of apoptosis comprises the method of Example 3, the correlation of results form the first and second timepoints determining any change in protease activity which indicates the onset of apoptosis.

Table 1 Mutants generated from GFP(S65T)

Mutant Name	Mutation site(s)	Site(s) mutated to SEQ ID NO:	Fluorescent Intensity	Cleavage by caspase-3
D1	V22	14	No	Not determined
D2	G33-G35	15	No	Not determined
D3	D102-D103	16	No	No
D4	E115-D117	17	3+	Partial
D5	F130-E132	14	No	Not determined
D6	F130-E132	18	+	No
D7	E172	15	3+	Partial
D8	D117-T118	19	2+	Partial
D9	D190-G191	30	3+	Partial
E2	T118	20	2.5+	Partial
E3-1	G134	21	2+	Partial
E3-5	G134	22	2+	Partial
E3-9	G134	23	2+	Partial
E3-12	G134	24	No	Partial
E4-a	V193	25	3+	Partial
E4-g	V193	26	3+	Partial
E4-j	V193	27	3+	Partial
E4-o	V193	28	2+	Partial
E4-p	V193	29	2+	Partial

The mutation sites referred to in Tables 1 and 2 are replaced in whole by the referenced SEQ ID NO. Thus for mutant D1, amino acid V22 is replaced by SEQ ID NO: 14. Similarly in mutant D8, amino acids D117 and T118 are replaced by by SEQ ID NO: 19.

The fluorescent intensity of each clone detailed in Tables 1 and 2 was normalized against the fluorescence of positive controls of GFP(S65T) whose intensity was rated as 5+.

Table 2 Mutants generated from a mammalian version of GFP (EGFP)

Mutant Name	Mutation site(s)	Site(s) mutated to SEQ ID NO:	Fluorescent Intensity	Cleavage by caspase-3
β6	F131-D134	31	No	Not determined
β9	G192-V194	14	No	Not determined
β10	P212-E214	14	3+	No
MD9	D191-G192	30	3+	Partial

Table 3 Caspases and their cleavage sites

Protease	Alterntive names	Recognition/cleavage sequence (SEQ ID NO:)	Protein substrate
caspase-1	ICE	7	Pro-IL-1β
caspase-2	ICH-1/ Nedd2	8	PARP
caspase-3	CPP32/ Yama/ apopain	4	PARP, DNA-PK, SREBP1,2, rho-GDI
caspase-4	TX/ ICH-2/ ICErel-II	9/10	
caspase-5	TY/ ICErel-III	9/10	
caspase-6	Mch2	11	Lamin A
caspase-7	Mch3/ ICE-LAP3/ CMH-1	4	PARP, pro-caspase 6, SREBP1,2
caspase-8	MACH/ FLICE/ Mch5	12	PARP
caspase-9	ICE-LAP6/ Mch6	13	PARP
caspase-10	FLICE2/ Mch4		
caspase-11	ICH-3		
caspase-12	DRONC		
caspase-13	ERICE		
caspase-14	MICE	4	

PARP: poly(ADP-ribose)polymerase

References: Cryns, V. and Yuan, J., 1998, Genes Dev., 11: 1551-1570; Cohen, G.M., 1997, Biochem. J., 326(pt1): 1-16; Negatta, S., 1997, Cell, 88: 355-365

CLAIMS

1. A fluorescent protein modified such that said modified fluorescent protein incorporates a cleavage site for a protease, cleavage of said modified fluorescent protein at said cleavage site by said protease causing the alteration of at least one of the emission and excitation spectra of said modified fluorescent protein.

2. A fluorescent protein according to claim 1, being a green fluorescent protein.

3. A fluorescent protein according to claim 2, said modified fluorescent protein having said cleavage site incorporated in the loop structure joining any pair of adjacent β-sheets.

4. A fluorescent protein according to claim 3, said pair of adjacent β-sheets being seleceted from the group consisting β-sheets numbers 9 and 10, 5 and 6, and 8 and 9.

5. A fluorescent protein according to claim 3, said modified fluorescent protein being selected from any one of the group consisting D9, MD9, D4, D7, D8, E2, E3-1, E3-5, E3-9, E3-12, E4-a, E4-g, E4-j, E4-o and E4-p.

6. A fluorescent protein according to claim 1, being selected from any one of the group consisting BFP, CFP, YFP and DsRed.

7. A fluorescent protein according to claim 1, said cleavage site having the sequence of any one of the group consisting SEQ ID NOs: 4 and 7-13.

8. A nucleic acid sequence encoding a fluorescent protein according to claim 1.

9. A recombinant DNA construct comprising a regulatory element operatively linked to a nucleic acid sequence according to claim 8.

10. A cell transformed or transfected with a recombinant DNA construct according to claim 9.

11. A method of determining protease activity in a sample cell transformed or transfected according to claim 10, comprising the steps of:

i) determining at least one of the emission and excitation spectra of said sample cell;

ii) determining for a cell transformed or transfected according to claim 9 and having a known protease activity the emission and/or excitation spectra determined in step (i);

iii) comparing the result of detection steps (i) and (ii); and

iv) correlating the results of comparison step (iii) to determine the level of said protease activity in said sample cell.

12. A method of detecting protease activity in a sample cell according to claim 11, the cell having a known protease activity having no protease activity.

13. A method of detecting a change in protease activity in a sample cell transformed or transfected according to claim 10, comprising the steps of:

i) at a first timepoint, determining at least one of the emission and excitation spectra of said sample cell;

ii) at a second timepoint, re-determining the emission and/or excitation spectra of said sample cell determined in step (i);

iii) comparing the results of steps (i) and (ii); and

iv) correlating the results of comparison step (iii) to determine any change in said protease activity in said sample cell.

14. A method of determining the effect of a compound on the activity of a protease in a cell transformed or transfected according to claim 10, comprising the steps of:

i) determining at least one of the emission and excitation spectra of said cell;

ii) treating said cell with said compound and re-determining the emission and/or excitation spectra of said cell determined in step (i);

iii) comparing the results of steps (i) and (ii); and

iv) correlating the results of comparison step (iii) to determine the effect of said test agent upon said activity of said protease.

15. A method of determining the effect of a compound on the activity of a protease in first and second cells transformed or transfected according to claim 10, comprising the steps of:

i) determining at leats one of the emission and excitation spectra of said first cell;

ii) treating said second cell with said compound and determining the emission and/or excitation spectra of said second cell determined in step (i) for said first cell;

iii) comparing the results of steps (i) and (ii); and

iv) correlating the results of comparison step (ii) to determine the effect of said compound upon said activity of said protease.

16. A method of determining protease activity in a sample, comprising the steps of:

i) adding to said sample a fluorescent protein according to claim 1 and the conditions necessary to allow fluorescence, and determining at least one of the emission and excitation spectra of said sample;

ii) adding to a control sample a fluorescent protein according to claim 1 and the conditions necessary to allow fluorescence, and determining the emission and/or excitation spectra determined in step (i), the control sample having a known protease activity;

iii) comparing the result of detection steps (i) and (ii); and

iv) correlating the results of comparison step (iii) to determine the level of said protease activity in said sample.

17. A method of detecting protease activity in a sample according to claim 16, the control sample not having any protease activity.

18. A method of detecting a change in protease activity in a sample, comprising the steps of:

i) adding to said sample a fluorescent protein according to claim 1 and the conditions necessary to allow fluorescence;

ii) at a first timepoint, determining at least one of the emission and excitation spectra of said sample;

iii) at a second timepoint, re-determining the emission and/or excitation spectra of said sample determined in step (i);

iv) comparing the results of steps (ii) and (iii); and

v) correlating the results of comparison step (iv) to determine any change in the level of said protease activity in said sample.

19. A method of determining the effect of a compound on the activity of a protease in a sample, comprising the steps of:

i) adding to said sample a fluorescent protein according to claim 1 and the conditions necessary to allow fluorescence;

ii) determining at least one of the emission and excitation spectra of said sample;

iii) treating said sample with said compound and re-determining the emission and/or excitation spectra of said sample determined in step (ii);

iv) comparing the results of steps (ii) and (iii); and

v) correlating the results of comparison step (iv) to determine the effect of said test agent upon said activity of said protease.

20. A method of determining the effect of a compound on the activity of a protease in first and second samples, comprising the steps of:

i) adding to said samples a fluorescent protein according to claim 1 and the conditions necessary to allow fluorescence;

ii) determining at leats one of the emission and excitation spectra of said first sample;

iii) treating said second sample with said compound and determining the emission and/or excitation spectra of said second sample determined in step (ii) for said first sample;

iv) comparing the results of steps (ii) and (iii); and

v) correlating the results of comparison step (iii) to determine the effect of said compound upon said activity of said protease.

21. The use of a cell according to claim 10 in a method of determining the activity of said protease.

22. The use of a protein according to claim 1 in a method of determining the activity of said protease.

23. The use of a protein according to claim 1 in a method of determining the effect of a compound on the activity of said protease.

24. The use of a protein according to claim 1 in a method of determining the effect of first and second compounds on the activity of said protease.

25. A fluorescent protein according to claim 1, said protease being a caspase.

26. A fluorescent protein according to claim 25, said caspase being selected form any one of the group of caspase-3, caspase-8 and caspase-9.

27. A method according to any one of claims 11-15, said protease comprising a caspase and said method comprising a method of detecting apoptosis of said sample cell.

ABSTRACT

Modified Fluorescent Proteins for Detecting Protease Activity

The present invention concerns fluorescent proteins modified such that said modified fluorescent protein incorporates a cleavage site for a protease, cleavage of said modified fluorescent protein at said cleavage site by said protease causing the alteration of at least one of the emission and excitation spectra of said modified fluorescent protein. In particular, the invention is concerned with using these modified fluorescent proteins as probes for detecting protease activity in living cells during the programmed cell death process (apoptosis).

Also provided are nucleic acid sequence encoding same, recombinant DNA constructs expressing same, cells transformed or transfected with same, methods for detecting protease activity, and methods of detecting agents which affect protease activity, and kits for same.

Figure 1

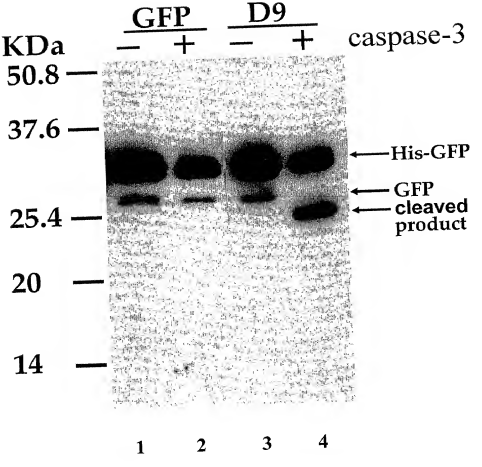
GFP
D9
KDa
– + – +
caspase-3
50.8
37.6
25.4
20
14
His-GFP
GFP
cleaved
product
1 2 3 4

Fig.2

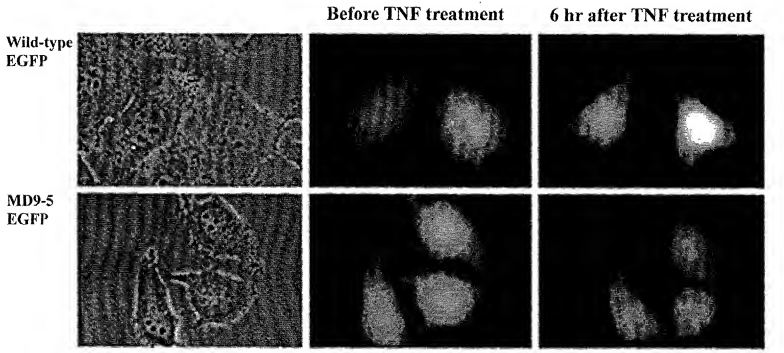

Figure 3

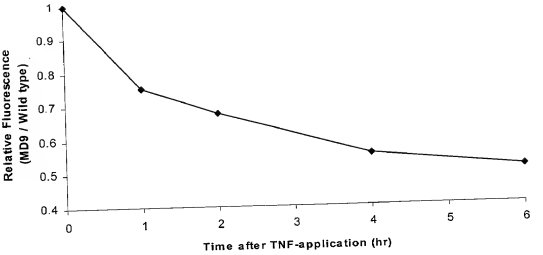
Relative Fluorescence
(MD9 / Wild type)
1
0.9
0.8
0.7
0.6
0.5
0.4
0
1
2
3
4
5
6
Time after TNF-application (hr)

Fig.4

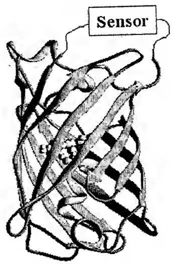

Figure 5

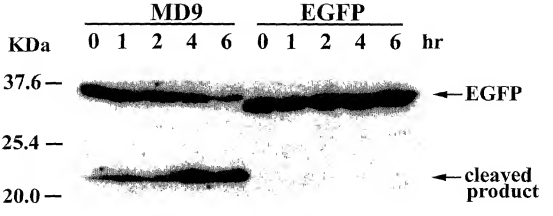

Fig.6

Locations of mutation sites in GFP 3D structure

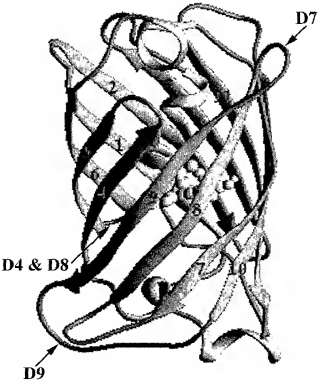

Fig.7

Attorney Docket No.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(includes reference to PCT International Applications)

As a below named inventor, I hereby declare that :

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

"Modified Green Fluorescent Proteins"

the specification of which

(X) is attached hereto.

( ) was filed on as
Application Serial No.
and was amended on

( ) was filed as PCT International Application
No. on and was
amended under PCT article 19 on

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to me to be material to patentability of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim priority benefits under title 35, United States Code, Section 119 of any US provisional or foreign application(s) for patent or inventor's certificate or of any PCT International Application(s) designating at least one country other than the United States of America listed below and have also identified below any provisional or foreign application for patent or inventor's certificate or any PCT International Application(s) designating at least one country other than the United States of America having a filing date before that of the application on which priority is claimed :

Prior Provisional or Foreign/PCT Application(s) (if PCT, indicate "PCT" under Country)

Priority Claimed

Number	Country	Date/Month/Year filed	
			( ) Yes ( ) No

I hereby claim the benefit under Title 35, United States Code Section 120 of any United States application(s) or PCT International Application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior application(s) in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulation, Section 1.56, which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application :

Application Serial No. or PCT Application No. (and any assigned USSN)	US or PCT Filing Date	Status -- Patented, Pending or Abandoned

POWER OF ATTORNEY : As a named Inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Wellington M Manning Jr.	Registration No. 22,376
Julian W Dority	Registration No. 20,268
James M Bagarazzi	Registration No. 29,609
Richard M Moose	Registration No. 31,226
Mark C Dukes	Registration No. 32,343
Stephen E Bondura	Registration No. 35,070
Neil C Jones	Registration No. 35,561
Craig N Killen	Registration No. 35,218
Timothy A Cassidy	Registration No. 38,024
Lloyd G Farr	Registration No. 38,446
Jeffrey M Karmilovich	Registration No. 35,915
Frances T Barnes	Registration No. 41,598
J Bennett Mullinax	Registration No. 36,221
Judy C Jarecki-Black	Registration No. 44,170

Send Correspondence to :
Attn. Judy C Jarecki-Black, PhD
Dority & Manning, PA
PO Box 1449
Greenville, SC 29602-1449

Direct Telephone Calls to :
Attn. Judy C Jarecki-Black, PhD
(864) 271 1592
FAX (864) 233 7342

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventor's Signature: D. C. Chang

Full name of Inventor: Donald Choy Chang

Date of Signature: 24 December, 1999

Residence and/or Post Office Address: Tower 7, Flat 2A, HKUST, Clear Water Bay, Kowloon, Hong Kong

Citizenship: USA

Inventor's Signature: Qian Luo

Full name of Inventor: Qian Luo

Date of Signature: 23-12-1999

Residence and/or Post Office Address: Biology department, The Hong Kong University of Science & Technology, Kowloon, Hong Kong

Citizenship: Canadian

SEQUENCE LISTING

<110> CHANG, Donald C
LUO, Qian

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<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: PCR primer

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1 5 10

<210> 3
<211> 39
<212> DNA
<213> Artificial Sequence

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1 5 10 15

gaa tta gat ggt gat gtt aat ggg cac aaa ttt tct gtc agt gga gag 96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
20 25 30

ggt gaa ggt gat gca aca tac gga aaa ctt acc ctt aaa ttt att tgc 144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
35 40 45

act act gga aaa cta cct gtt cca tgg cca aca ctt gtc act act ttc 192
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
50 55 60

act tat ggt gtt caa tgc ttt tca aga tac cca gat cat atg aaa cag 240
Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln
65 70 75 80

cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa aga 288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
85 90 95

act ata ttt ttc aaa gat gac ggg aac tac aag aca cgt gct gaa gtc 336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
100 105 110

aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt att 384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
115 120 125

gat ttt aaa gaa gat gga aac att ctt gga cac aaa ttg gaa tac aac 432
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
130 135 140

tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat gga 480
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145 150 155 160

atc aaa gtt aac ttc aaa att aga cac aac att gaa gat gga agc gtt 528
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
165 170 175

caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc cct 576
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
180 185 190

gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt tcg 624
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
195 200 205

aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt gta 672
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
210 215 220

aca gct gct ggg att aca cat ggc atg gat gaa cta tac aaa taataa 720
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225 230 235

<210> 6
<211> 238
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Possible
cleavage site

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1 5 10 15

Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
20 25 30

Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
35 40 45

Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
50 55 60

Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln
65 70 75 80

His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
85 90 95

Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
100 105 110

Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
115 120 125

Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
130 135 140

Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145 150 155 160

Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
165 170 175

Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
180 185 190

Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
195 200 205

Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
210 215 220

Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225 230 235

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<400> 7
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<210> 8
<211> 4
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<400> 8
Asp Glu His Asp
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<210> 9
<211> 4
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<220>
<223> Description of Artificial Sequence: Possible cleavage site

<400> 9
Trp Glu His Asp
1

<210> 10
<211> 4
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<400> 10
Leu Glu His Asp
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<210> 11
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<400> 11
Val Glu Ile Asp
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<210> 12
<211> 4
<212> PRT
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<220>
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<400> 12
Leu Glu Thr Asp
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<210> 13
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cleavage site

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Leu Glu His Asp
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<210> 14
<211> 3
<212> PRT
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cleavage site

<400> 14
Glu Val Asp
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<210> 15
<211> 3
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cleavage site

<400> 15
Asp Glu Val
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<210> 16
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cleavage site

<400> 16
Asp Glu Asp Asp
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<210> 17
<211> 5

<212> PRT
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<400> 17
Asp Glu Val Asp Gly
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<210> 18
<211> 2
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<400> 18
Glu Val
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<210> 19
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<400> 19
Asp Glu Val Asp Gly Thr
1 5

<210> 20
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<400> 20
Glu Val Asp Gly Pro
1 5

<210> 21

<211> 5
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cleavage site

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1 5

<210> 22
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cleavage site

<400> 22
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1 5

<210> 23
<211> 5
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cleavage site

<400> 23
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1 5

<210> 24
<211> 5
<212> PRT
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cleavage site

<400> 24
Glu Val Asp Gly Gly
1 5

<210> 25
<211> 6
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cleavage site

<400> 25
Ala Asp Glu Val Asp Ile
1 5

<210> 26
<211> 6
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cleavage site

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1 5

<210> 27
<211> 6
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cleavage site

<400> 27
Thr Asp Asp Val Asp Leu
1 5

<210> 28
<211> 6
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cleavage site

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Trp Asp Glu Val Asp Ala
1 5

<210> 29
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<212> PRT
<213> Artificial Sequence

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cleavage site

<400> 29
Arg Asp Glu Val Asp Phe
1 5

<210> 30
<211> 5
<212> PRT
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<220>
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cleavage site

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1 5

<210> 31
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Possible
cleavage site

<400> 31
Glu Val Asp Gly
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<210> 32
<211> 239
<212> PRT
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<220>
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mammalian enhanced GFP

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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
            20                  25                  30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145                 150                 155                 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
        195                 200                 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225                 230                 235
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